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INWARD CURRENT CAUSED BY SODIUM-DEPENDENT UPTAKE OF GABA IN THE CRAYFISH STRETCH RECEPTOR NEURONE

BY K. KAILA*, B. RYDQVIST†, M. PASTERNACK* AND J. VOIPIO*

*From the *Department of Zoology, Division of Physiology, University of Helsinki, Arkadiankatu 7, SF-00100 Helsinki, Finland and the †Department of Physiology II, Karolinska Institutet, S-10410 Stockholm, Sweden*

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SUMMARY

1. A two-microelectrode current–voltage clamp and Cl^--selective microelectrodes were used to examine the effects of γ-aminobutyric acid (GABA) on membrane potential, current and intracellular Cl^- activity (a^i_{Cl}) in the crayfish stretch receptor neurone. All experimental solutions were CO_2–HCO_3^- free.

2. GABA (500 μM) produced a mono- or biphasic depolarization (amplitude $\leqslant$ 10 mV), often with a prominent initial depolarizing component followed by a transient shift to a more negative level. In some neurones, an additional depolarizing phase was seen upon washout of GABA. Receptor desensitization, being absent, played no role in the multiphasic actions of GABA.

3. The pronounced increase in membrane conductance evoked by GABA (500 μM) was associated with an increase in a^i_{Cl} which indicates that the depolarizing action was not due to a current carried by Cl^- ions.

4. The currents activated by GABA under voltage clamp conditions were inwardly directed when recorded at the level of the resting membrane potential, and they often revealed a biphasic character. The reversal potential of peak currents activated by pulses of 500 μM-GABA (E_{GABA}) was 9–12 mV more positive than the reversal potential of the simultaneously measured net Cl^- flux (E_{Cl}). E_{Cl} was 2–7 mV more negative than the resting membrane potential.

5. E_{GABA} (measured using pulses of 500 μM-GABA) was about 10 mV more positive than the reversal potential of the current activated by 500 μM-muscimol, a GABA agonist that is a poor substrate of the Na^+-dependent GABA uptake system.

6. In the absence of Na^+, the depolarization and inward current caused by 500 μM-GABA were converted to a hyperpolarization and to an outward current. Muscimol produced an immediate outward current both in the presence and absence of Na^+.

7. Following block of the inhibitory channels by picrotoxin (100–200 μM), the depolarizing effect of 500 μM-GABA was enhanced and the transient hyperpolarizing shifts were abolished.

8. In the presence of picrotoxin, GABA ($\geqslant$ 2 μM) produced a concentration-dependent monophasic inward current which had a reversal potential of +30 to +60 mV. This current was inhibited in the absence of Na^+ and by the GABA uptake blocker, nipecotic acid. Unlike the channel-mediated current, the picrotoxin-

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insensitive current was activated without delay also at low (2–10 μM) concentrations of GABA.

9. Brief ($\leqslant$ 10 s) pulses of GABA at a low concentration ($\leqslant$ 100 μM) produced only a small increase in conductance, and the reversal potential of the GABA-activated current obtained in this manner was close to that seen in the presence of picrotoxin. In contrast to this, the reversal potential of the current activated by pulses of 50 μM-muscimol was identical to that observed at 500 μM.

10. The present results indicate that a sodium-dependent electrogenic GABA uptake mechanism has a direct influence on the current and voltage responses evoked by GABA in the crayfish stretch receptor neurone. The current component attributable to uptake makes E_{GABA} significantly more positive than E_{Cl} and explains the mono- and biphasic depolarizing actions of GABA. When measured using low concentrations and short pulses of GABA, the preferential activation of the uptake mechanism leads to an estimate of E_{GABA} which is close to the reversal potential of the uptake current.

INTRODUCTION

A sodium-dependent high affinity system for the uptake of γ-aminobutyric acid (GABA) has been shown to be present in neuronal and glial cells of both vertebrates and invertebrates (reviews: Atwood, 1976; Krnjević, 1984; Erecińska, 1987; Kanner & Schuldiner, 1987; Dingledine, Boland, Chamberlin, Kawasaki, Kleckner, Traynelis & Verdoorn, 1988). It is generally agreed that clearance of GABA from the interstitial fluid in the nervous system in face of the activity of numerous GABAergic neurones is attributable to this uptake mechanism. Accordingly, inhibition of the GABA uptake system has been shown to lead to a marked potentiation of the actions of exogenously applied GABA (Curtis, Game & Lodge, 1976; Deisz & Dose, 1983; Brown & Scholfield, 1984; Rovira, Ben-Ari & Cherubini, 1984; Korn & Dingledine, 1986; Akaike, Maruyama, Sikdar & Yasui, 1987).

When first identified at the lobster neuromuscular junction, the Na^+-dependent GABA uptake system was tentatively assigned a role in terminating the postsynaptic action of neuronally released GABA (Iversen & Kravitz, 1968). The results pertinent to this topic are, however, controversial. In some cases, inhibitors of GABA uptake have been shown to produce an enhancement of the inhibitory postsynaptic current (Rekling, Jahnsen & Laursen, 1990), while in others, such an effect has not been detected (Curtis *et al.* 1976; Lodge, Johnston, Curtis & Brand, 1977; Horwitz & Orkand, 1980; Deisz *et al.* 1984; Brown & Scholfield, 1984).

An alteration in the availability of GABA at the inhibitory receptor channels is, however, not the only immediate postsynaptic effect that might be exerted by the GABA uptake mechanisms. From an electrophysiological point of view it is interesting that the uptake of GABA is electrogenic, being driven by the transmembrane Na^+ electrochemical gradient (Kanner & Schuldiner, 1987). This raises the possibility that the uptake mechanism might directly contribute to the postsynaptic potential changes caused by GABA (cf. Krnjević, 1984).

In this work we have examined the effects of GABA on membrane potential, current and on net movements of Cl^- in the crayfish stretch receptor neurone (SRN). The experiments were carried out under HCO_3^--free conditions (see Kaila,

Pasternack, Saarikoski & Voipio, 1989; Voipio, Pasternack, Rydqvist & Kaila, 1991) using a two-microelectrode current–voltage clamp and Cl^--selective microelectrodes. We show that, in addition to the well-known increase in Cl^- conductance, GABA (but not muscimol) induces a picrotoxin-insensitive inward current with a reversal potential of +30 to +60 mV. This current is blocked by the specific GABA uptake inhibitor nipecotic acid (Krogsgaard-Larsen, 1980) and by removal of extracellular Na^+, which suggests that it is caused by the operation of an electrogenic GABA uptake system in the SRN. The current component attributable to uptake makes the reversal potential of the GABA-induced total current significantly more positive than E_{Cl} (the Cl^- equilibrium potential), and explains the depolarizing action of GABA seen under HCO_3^--free conditions. A direct influence of the uptake system on GABA-induced voltage and current responses has not been previously demonstrated in a neurone which receives a GABAergic inhibitory innervation, such as the SRN (Kuffler & Edwards, 1958; Elekes & Florey, 1987).

METHODS

The experiments were carried out on the slowly adapting stretch receptor neurone of the crayfish *Astacus astacus* (see Brown, Ottoson & Rydqvist, 1978; Rydqvist & Zhou, 1989). The stretch receptor with its muscle was isolated and pinned on the bottom of a flow-through chamber, which was earthed by means of a conical agar–3 M-KCl bridge (Strickholm, 1968) placed at the downstream end. In a number of experiments, the stretch receptor neurone was impaled by three microelectrodes, two conventional ones for voltage or current clamping and a Cl^--selective microelectrode. All experiments were carried out at room temperature (20–21 °C).

Under control conditions, the neurones had a resting potential from −62 to −75 mV, action potentials with an overshoot of 20–30 mV and an input resistance ranging from 1·5 to 4 MΩ.

Conventional microelectrodes. Dry-bevelled microelectrodes (Kaila & Voipio, 1985) filled with 0·62 M-K_2SO_4 + 8·0 mM-KCl were used for measuring membrane potentials and passing current. They had a resistance of 10–40 MΩ.

Ion-selective microelectrodes. The Cl^--selective microelectrodes were constructed and calibrated as described in detail previously (Kaila *et al.* 1989). Briefly, dry-bevelled micropipettes pulled from tubing without a fibre (GC150, Clark Electrochemical, Pangbourne, Reading, UK) were silanized and back-filled with 100 mM-NaCl + 20 mM-HEPES (pH 7·6). A short column of the Cl^- sensor (Corning 477913) was taken into the tip. The resistance of these electrodes was about 10 GΩ. In those recordings (Fig. 3) which are related to measurements of the reversal potential of the GABA-induced Cl^- net flux, the differential signal of the Cl^--selective microelectrode (V_{Cl}^{diff}) is defined as

$$V_{Cl}^{diff} = V_{Cl} - V_m,$$

where V_{Cl} is the voltage recorded by the Cl^- electrode and V_m is the intracellular reference electrode voltage. (In a previous paper (Kaila *et al.* 1989), the differential signal was shown with inverted polarity which, of course, does not affect the value of the measured reversal potential.) The Cl^- activity coefficient was taken as 0·74 (Rauen, 1964).

Voltage and current clamp. Membrane currents, conductances and the reversal potential of currents activated by GABA and muscimol were measured using a two-microelectrode voltage clamp. In voltage clamp experiments (except when measuring reversal potentials), the membrane potential was held at a level equal to the resting membrane potential. In a few experiments, GABA-induced changes in membrane conductance were monitored by means of a current clamp.

Solutions. The crayfish saline contained (mM): NaCl, 207; KCl, 5·4; $CaCl_2$, 13·5; $MgCl_2$, 2·6; HEPES, 10 (pH 7·4; adjusted with NaOH). Na^+-free solutions were made by isomolar substitution of Na^+ by choline. In the latter solutions, adjustment of pH was made using the corresponding free base.

Drugs. GABA, nipecotic acid and muscimol were added from 50–100 mM stock solutions made in water. The picrotoxin stock solution was made in dimethylsulphoxide (DMSO). The final concentration of DMSO was 0·01–0·02 %. All drugs were from Sigma.

RESULTS

Effect of GABA on membrane potential

Exposure of the stretch receptor neurone to 500 μM-GABA produced a depolarization in the majority of the preparations examined (cf. Kuffler & Edwards, 1958; Voipio *et al.* 1991). The depolarizations were either purely monophasic or biphasic, with a prominent initial depolarizing component which was followed by a transient shift to a more negative level (see specimen recordings *a–c* in Fig. 1*A*). In some cases (six preparations out of forty-two), this negative shift transgressed the resting level thereby giving rise to a transient hyperpolarization (e.g. Fig. 1*Ac*). As shown below, the currents activated by GABA under voltage clamp conditions were inwardly directed when recorded at the level of the resting membrane potential, and they often revealed a similar biphasic character.

In some preparations, receptor desensitization has been shown to play a role in shaping the voltage and current responses evoked by exogenous GABA (Krnjević, 1981). However, current and voltage clamp measurements showed that no desensitization takes place in the SRN even during a prolonged application of 500 μM-GABA (cf. Fig. 2; and Deisz & Dose, 1983).

In some neurones, a clear depolarizing shift was observed upon washing off GABA (e.g. Fig. 1*Ac*). Evidence will be presented below to support the conclusion that the initial depolarizing effect as well as the 'off response' are due to an electrogenic uptake mechanism which operates at lower concentrations of applied GABA than those required for the activation of the inhibitory anion channels.

In Fig. 1*B*, the peak change in membrane potential (range from -5 to $+10$ mV) seen in forty-two neurones during a 30 s application of 500 μM-GABA is plotted as a function of the resting membrane potential. The peak depolarizations are indicated with filled circles and the transient hyperpolarizations observed in some preparations with open circles. It is evident from Fig. 1*B* that, under control conditions, there is no correlation between the resting membrane potential of a given neurone and its voltage response to GABA.

Effect of GABA on intracellular Cl^-

A conventional explanation to account for a depolarizing action of GABA is that Cl^- ions are actively accumulated in the target cell, which makes E_{Cl} more positive than the resting membrane potential (see Alvarez-Leefmans, 1990; Kaila & Voipio, 1990). However, direct evidence ruling out Cl^- as the carrier of the depolarizing current in the SRN was obtained in experiments of the kind shown in Fig. 2. Here, we examined simultaneously the effects of GABA on membrane potential, conductance (current clamp) and on the intracellular Cl^- activity (a^i_{Cl}). A consistent finding was that the GABA-induced depolarization was associated with an increase in a^i_{Cl}. This is a crucial observation since it clearly indicates that the depolarization caused by GABA cannot be due to a current carried by Cl^-. The GABA-induced increase in a^i_{Cl} most likely reflects a redistribution of Cl^- ions mediated by the inhibitory channels. With 500 μM-GABA, the chloride redistribution (as measured on the basis of the change in V^{diff}_{Cl}; see Methods) had a half-time which ranged from 15 to 30 s ($n = 6$).

The depolarizing effect of GABA was not attributable to a channel-mediated efflux

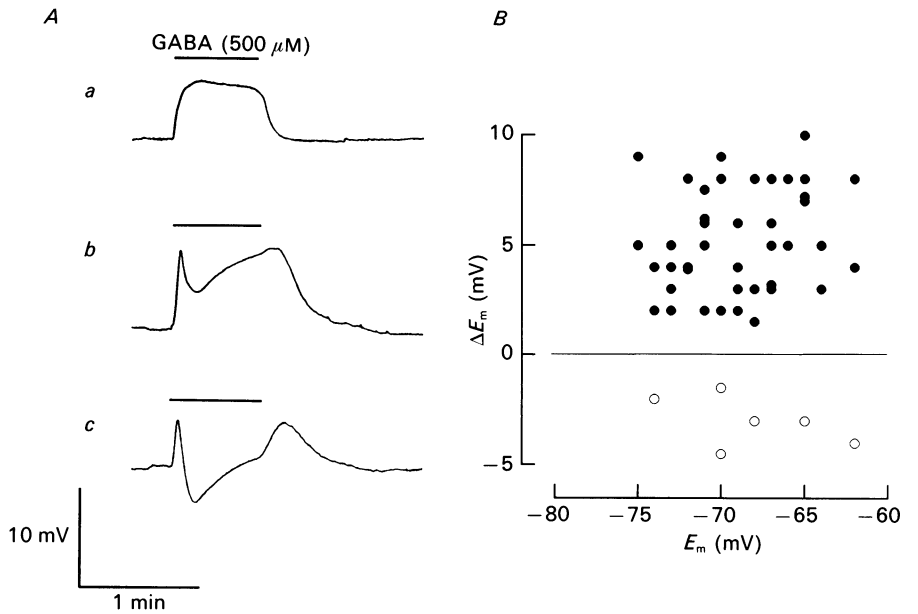

Fig. 1. Effect of GABA (500 μM) on membrane potential. *A*, specimen recordings (*a*, *b*, *c*) from three neurones. *B*, peak change in membrane potential (ΔE_m) observed during a 30 s application of GABA in forty-two neurones, plotted as a function of the resting membrane potential (E_m). ●, peak depolarizations; ○, peak hyperpolarizations.

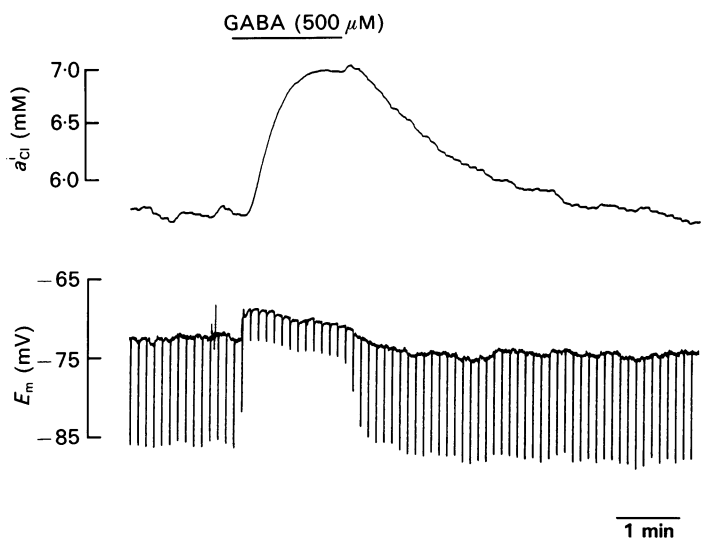

Fig. 2. Effect of GABA (50 μM) on membrane potential, input resistance and on intracellular Cl^-. The amplitude of the hyperpolarizing current pulses was 5 nA.

of bicarbonate (Kaila *et al.* 1989) since the present experiments were carried out in the nominal absence of CO_2–HCO_3^-. Evidently, the influx of Cl^- which leads to the rise in a^i_{Cl} opposes the effect of an as yet unidentified depolarizing mechanism.

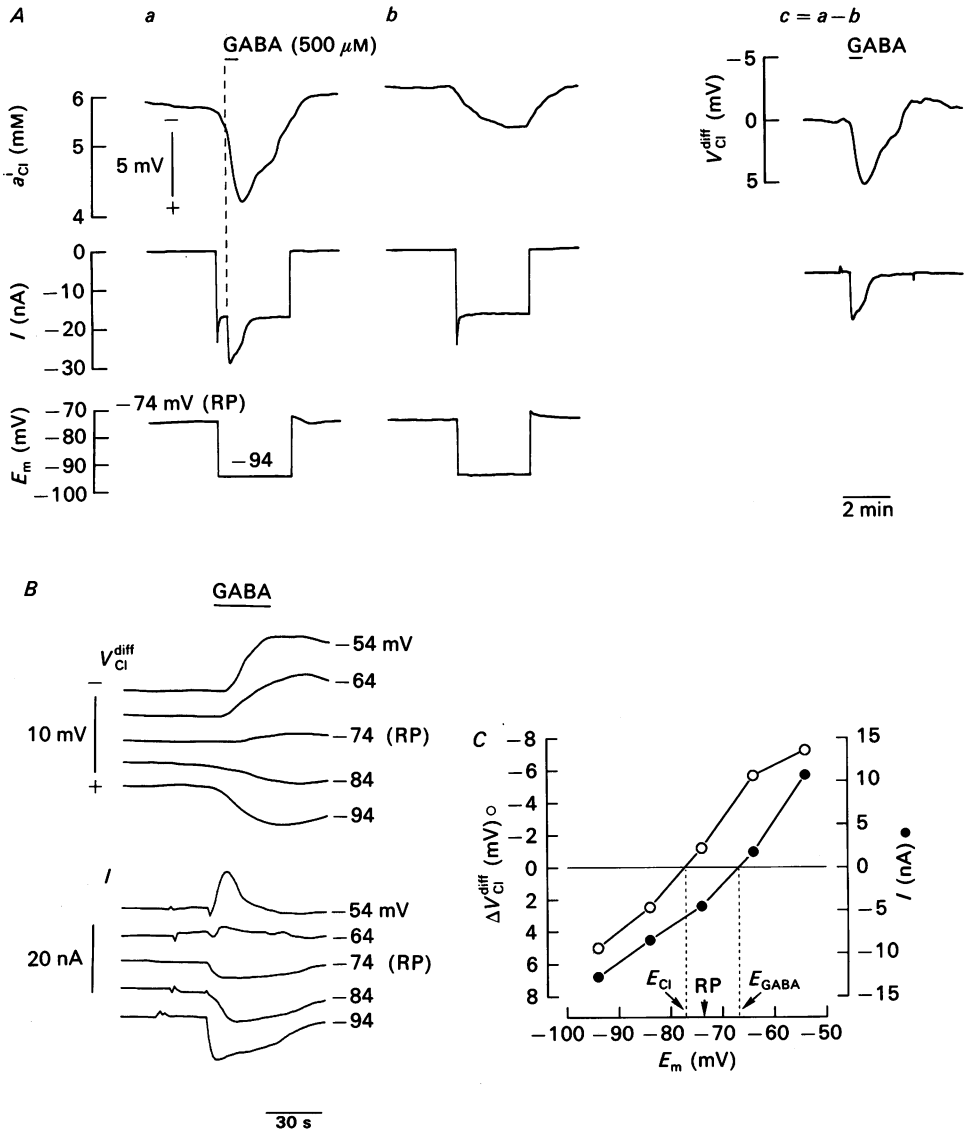

Fig. 3. Simultaneous measurement of the reversal potential of GABA-activated current (E_{GABA}) and of the GABA-induced net flux of Cl^- (E_{Cl}). *Aa*, effect of a step in membrane potential from −74 to −94 mV, followed by a 30 s pulse of 500 μM-GABA, on a_{Cl}^i and on membrane current (RP, resting membrane potential; −74 mV). The 5 mV calibration bar applies to the differential voltage (V_{Cl}^{diff}) of the Cl^--selective electrode. *Ab*, influence of clamping at −94 mV on membrane current and on intracellular Cl^- in the absence of GABA. *Ac* (=*Aa*−*Ab*), effect of GABA only on V_{Cl}^{diff} and on current. *B*, effect of GABA on V_{Cl}^{diff} and on membrane current at holding potentials from −54 to −94 mV. *C*, peak currents and changes in V_{Cl}^{diff} produced by GABA plotted against membrane potential. Note that $E_{Cl} < RP < E_{GABA}$. Data shown from a single continuous experiment.

Simultaneous measurement of the reversal potential of the GABA-induced current and of the GABA-induced net chloride flux

The observation that the depolarization caused by GABA is linked to an increase in a_{Cl}^{i} implies that E_{GABA} does not equal E_{Cl} and, more specifically, $E_{GABA} > E_{Cl}$. The

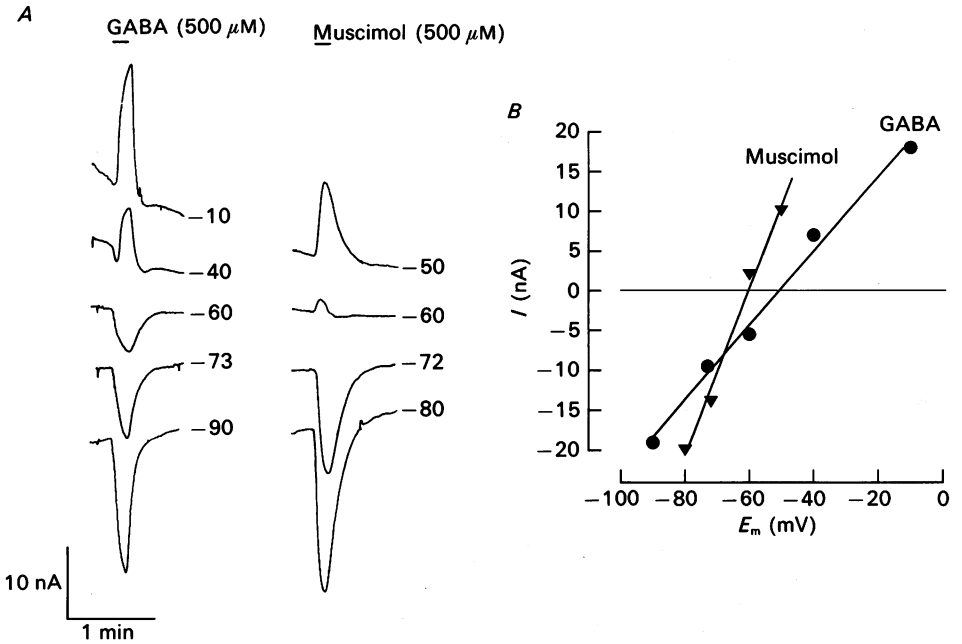

Fig. 4. Comparison of the reversal potentials of currents activated by 10 s pulses of GABA (500 μM) and muscimol (500 μM). *A*, specimen recordings of currents (figures indicate holding potentials in mV). *B*, *I–V* plot of peak currents; data from *A*.

quantitative relationship between E_{GABA} and E_{Cl} was examined more closely under voltage clamp conditions by comparing the reversal potential of the current activated by a 30 s pulse of bath-applied GABA (500 μM) and the reversal potential of the simultaneous net flux of Cl^- measured by a Cl^--selective microelectrode. As discussed in detail elsewhere (Kaila *et al.* 1989), measuring the reversal potential of the channel-mediated net flux of Cl^- by means of a Cl^--selective microelectrode gives an estimate of E_{Cl} which does not rely on the absolute calibration of the electrode (Kaila *et al.* 1989). The reversal potential of the channel-mediated net flux of Cl^- (which must equal E_{Cl}) can be obtained by estimating the reversal level of the GABA-induced change in the differential voltage of the Cl^- electrode (V_{Cl}^{diff}; see Methods). At E_{Cl}, the opening of Cl^--permeable channels will not induce a net movement of chloride, and hence there is no change in V_{Cl}^{diff}.

The principle of these measurements is illustrated in Fig. 3*A*. As shown in part *b* of Fig. 3*A*, clamping at a rather negative membrane potential (−94 mV) produces a slight decrease in a_{Cl}^{i} which probably reflects the background chloride conductance of the resting neurone. A pulse of GABA applied while holding at −94 mV (Fig. 3*Aa*) produces an inward current and a rapid positive shift in the differential voltage of the

Cl^--selective electrode indicative of a fall in a^i_{Cl} (i.e. of a net efflux of Cl^-). The quantitative effect of GABA on membrane current and on V^{diff}_{Cl} can be obtained by subtracting the latter recording from the former one (Fig. 3*Ac*).

Figure 3*B* shows a family of recordings similar to those in part *c* of Fig. 3*A*, obtained at various holding potentials. In order to minimize voltage-dependent changes in a^i_{Cl}, the membrane potential was not clamped during the intervals between individual measurements. A notable observation is that the Cl^- component of the GABA-induced current reverses at a level more negative than the resting membrane potential (RP; −74 mV) while the GABA-induced total current reverses at a voltage level more positive than the resting potential. This difference is more clearly shown in Fig. 3*C*, where the peak currents and changes in V^{diff}_{Cl} produced by GABA have been plotted as a function of membrane potential. Four experiments of this kind showed that the reversal potential of the GABA-activated total current (E_{GABA}) was 9–12 mV more positive than the reversal potential of the current component which is carried by Cl^- (i.e. E_{Cl}).

The observation that E_{Cl} is more negative (by 2–7 mV; $n = 4$) than the resting membrane potential is consistent with the presence of a K^+–Cl^- co-transport mechanism in the SRN (Deisz & Lux, 1982; Aickin, Deisz & Lux, 1982). This means that if the action of GABA were solely attributable to an increase in Cl^- conductance, GABA should produce an outward (hyperpolarizing) current at the level of the resting membrane potential. Very interestingly, such an effect was characteristic of the specific $GABA_A$ agonist, muscimol (see below).

In conclusion, the above results indicate that in a resting neurone, GABA activates an outward current carried by Cl^- and, simultaneously, an inward current of unknown ionic nature. The experiments that will be described below were designed to identify the inwardly directed component of the total current. In the following, the expression 'inward current' implies that the recording has been made at a holding potential which equals the resting membrane potential of the neurone under study.

Comparison of the reversal potentials of currents activated by GABA and by muscimol

Cl^- and HCO_3^- are the only physiologically relevant ions that are permeant in GABA-gated anion channels (Kaila *et al.* 1989; Kaila & Voipio, 1990). However, the results described so far indicate that the inward current activated by GABA does not involve either of these two anions. In order to test the idea that an electrogenic uptake system contributes to the actions of GABA, we compared the reversal potentials of the currents activated by GABA and by muscimol, a specific agonist of GABA-gated anion channels which is a poor substrate of Na^+-dependent uptake (Nistri & Constanti, 1979; Krause, Ikeda & Roberts, 1981; Dingledine *et al.* 1988; Deisz & Dose, 1983).

Experiments of the type illustrated in Fig. 4 showed that the reversal potential of the current activated by 500 μM-muscimol was 8–10 mV ($n = 3$) more negative than that activated by 500 μM-GABA. Such a difference in the reversal potentials of the currents activated by the two agonists is consistent with the assumption that the GABA-induced current is composed of two components: one mediated by the inhibitory anion channels and one by an uptake mechanism. This hypothesis gained strength in the experiments to be described below.

The slopes of the *I–V* plots in Fig. 4*B* also indicate that the conductance activated by muscimol pulses was larger than that induced by GABA. This difference is in agreement with the higher apparent potency of muscimol as an agonist of GABA-gated anion channels in the SRN (Krause *et al.* 1981). However, it is important to point out in this context that the difference in the reversal potentials of the currents activated by high equimolar (500 μM) concentrations of GABA and muscimol is not due to the higher conductance activated by the latter drug. This was verified in experiments which showed that, in contrast to observations made with GABA (see below; Fig. 8), the reversal potential of the current responses evoked by 50–500 μM-muscimol was not concentration dependent.

Dependence on sodium of the GABA-induced inward current

In various cells, electrogenic uptake of GABA is driven by the Na^+ electrochemical gradient (Kanner & Schuldiner, 1987; Erecińska, 1987). Very interestingly, we found that under Na^+-free conditions, the depolarization and inward current caused by GABA were converted to a hyperpolarization and to an outward current (Fig. 5*A* and *B*). In contrast to this, muscimol induced an immediate outward current both in the presence and absence of Na^+ (Fig. 5*C*).

Since the GABA-induced current shown in Fig. 5*B* is recorded at one potential only, one might argue that the reversal of its polarity could be due to a large junction potential change caused by the complete replacement of Na^+ by choline. However, when switching to the choline solution, the conical agar–3 M-KCl bridge used for earthing the experimental chamber (see Methods) produced a very small voltage transient (0·5–1·5 mV; duration less than 15 s) as measured using a flowing 3 M-KCl electrode–conical electrode pair (cf. Strickholm, 1968). In addition, using the Henderson equation with values for the relevant ionic activity coefficients and mobilities (Robinson & Stokes, 1955; Rauen, 1964; Spivey & Snell, 1964; Macaskill, Mohan & Bates, 1977; Ammann, 1986), we obtained an estimate of 0·7 mV for the shift in bath potential upon application of the Na^+-free solution, which is clearly too small to have a significant influence on the present measurements.

Influence of picrotoxin and nipecotic acid on the actions of GABA

The above results strongly suggest that the depolarizing inward current induced by GABA is not mediated by the inhibitory anion channels. To further examine this assumption, a series of experiments were performed using picrotoxin (PTX), a non-competitive inhibitor of GABA-gated anion channels (Takeuchi & Takeuchi, 1969; Nistri & Constanti, 1979; Aickin, Deisz & Lux, 1981).

Rather strikingly, we found that, despite the fact that 100–200 μM-PTX produces a near-complete inhibition of the GABA-activated conductance (Takeuchi & Takeuchi, 1969), the drug did not attenuate, but rather enhanced, the inward current. In experiments where GABA had a clear biphasic action, PTX abolished the transient outward shift in current which took place after the initial inwardly directed component (Fig. 6*A*).

As was to be expected on the basis of the effects of the sodium-free solution described above (see Fig. 5*A* and *B*), the PTX-insensitive inward current was completely blocked in the absence of Na^+. Both the insensitivity to PTX and sensitivity to extracellular sodium are consistent with the hypothesis that the GABA-induced inward current is mediated by an uptake mechanism. Further evidence favouring this view was gained in experiments with nipecotic acid.

Nipecotic acid (NPA) is a widely used inhibitor of the Na^+-dependent GABA uptake mechanism (Krogsgaard-Larsen, 1980). In agreement with previous reports

(Krause *et al.* 1981; Deisz & Dose, 1983), we found that, in the absence of PTX, 500 μM-NPA enhanced the action of GABA on membrane conductance and slowed down the attainment of the resting conductance level upon washoff of GABA (not illustrated). In contrast to the facilitation of the GABA-induced Cl^- conductance, the PTX-insensitive inward current was blocked in the presence of NPA (Fig. 6*B*).

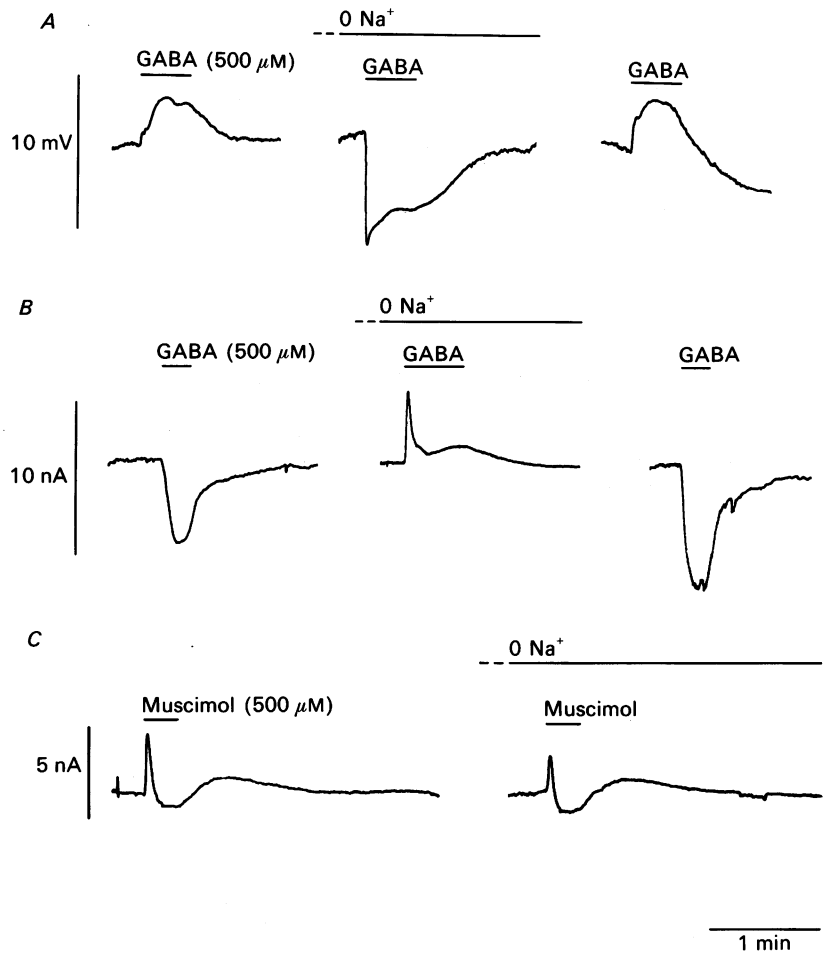

Fig. 5. Role of sodium in the actions of GABA and muscimol. Effect of GABA (500 μM) on membrane potential (*A*) and on membrane current (*B*) in the presence and absence of Na^+ (in this and subsequent figures, dashed line indicates pre-exposure of > 30 s). *C*, absence of an influence of Na^+ on the polarity of the current activated by 500 μM-muscimol. In *B* and *C*, holding potential equals resting membrane potential.

Consistent with the competitive nature of the inhibition action of NPA on GABA uptake (Krogsgaard-Larsen, 1980), an inward current of 0·25–2·5 nA was observed upon the application of NPA (500–1000 μM) in each of the five preparations tested (Fig. 6*B*). The idea that this effect was, indeed, mediated by the uptake mechanism gained support from the finding that NPA failed to produce an inward current in the absence of Na^+.

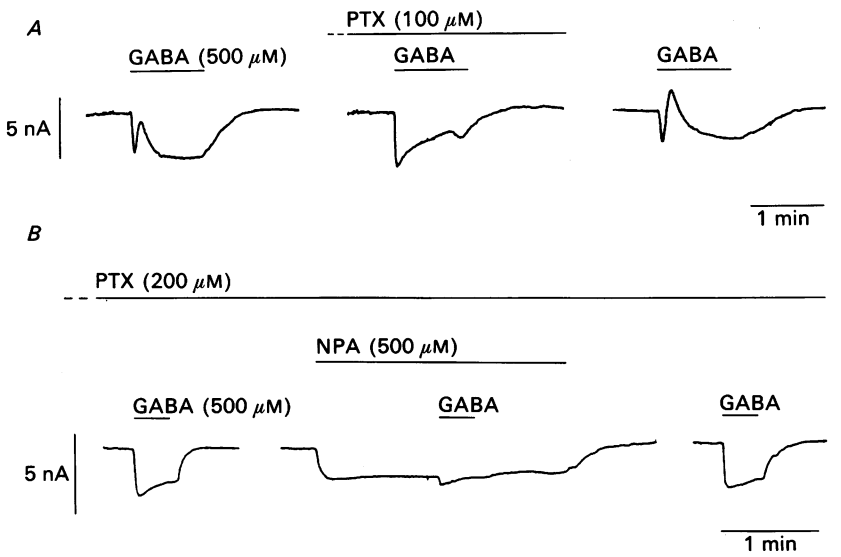

Fig. 6. Influence of picrotoxin (PTX; 100–200 μM) and of nipecotic acid (NPA; 500 μM) on the actions of GABA (500 μM). *A*, effect of PTX on GABA-induced changes in holding current measured while clamping at the resting membrane potential. Note block of the transient outward shift and increase in peak amplitude of the inward component in the presence of PTX. *B*, inhibition by NPA of the PTX-insensitive inward current activated by GABA. Recordings in *A* and *B* are from separate neurones.

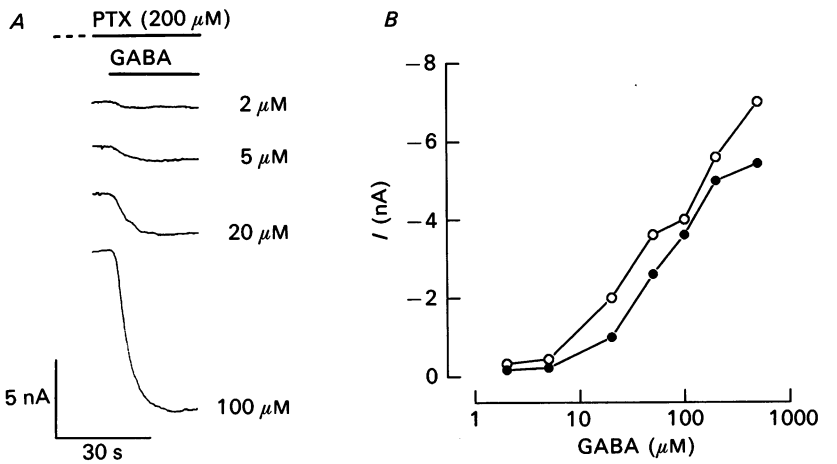

Fig. 7. Concentration dependence of the inward current activated by GABA in the presence of 200 μM-PTX. *A*, specimen recordings obtained using 2–100 μM-GABA. *B*, peak value of PTX-insensitive inward current plotted as a function of concentration; data from two neurones.

Concentration dependence of the putative GABA uptake current

The dependence on GABA concentration of the inward current component was examined in the presence of 200 μM-PTX. As shown in Fig. 7, the PTX-insensitive inward current was detectable at very low (2 μM) concentrations of GABA and it

attained its maximum (5–12 nA; $n = 4$) at 200–500 μM. True saturation of the putative uptake current could not be demonstrated under the present conditions since at high concentrations of GABA ($\geqslant 500$ μM) a decrease in the peak inward current, which was paralleled by an increase in conductance, took place. The fall in the peak amplitude of the inward current at high concentrations of GABA was most likely due to the activation of a small fraction of the GABA-sensitive Cl^- channels

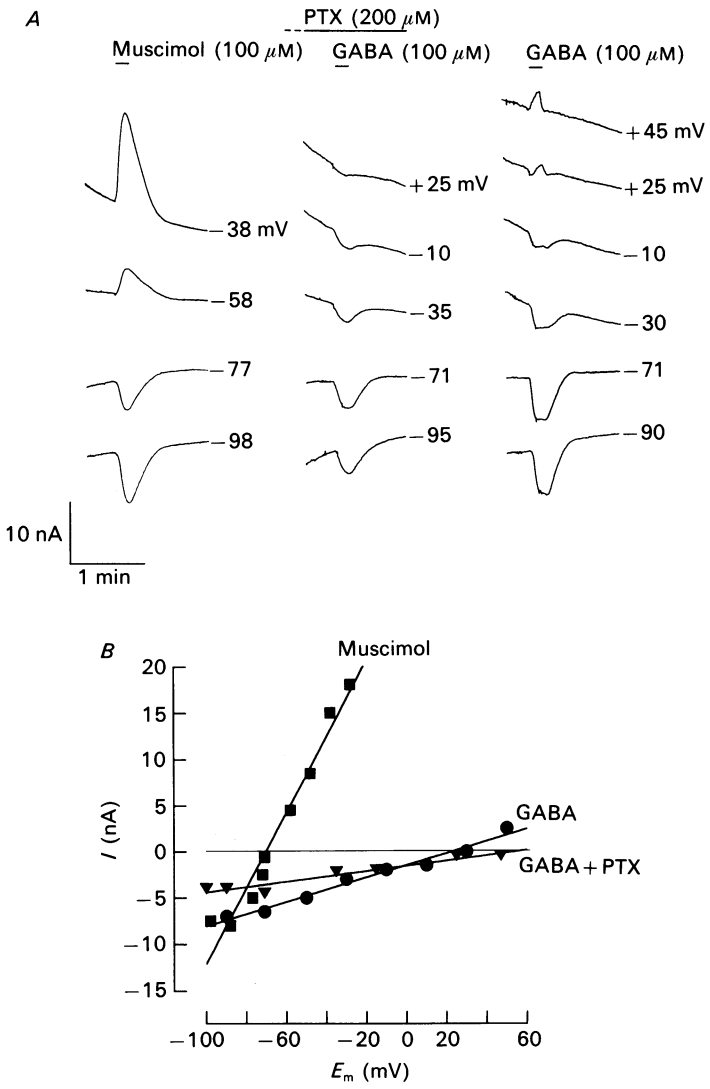

Fig. 8. Measurement of the reversal potential of currents activated by 10 s pulses of 100 μM-GABA and 100 μM-muscimol under control conditions and by 100 μM-GABA in the presence 200 μM-PTX. *A*, specimen recordings of currents. *B*, current–voltage relationships of peak responses.

(and, hence, of a Cl^--mediated outward current) despite the presence of PTX (Takeuchi & Takeuchi, 1969).

A notable feature of the inward current is that it was activated without delay following application of GABA even when using very low concentrations, i.e. 2–10 μM (Fig. 7*A*). This contrasts with the concentration-dependent delay of the channel-mediated effects of GABA (Deisz & Dose, 1983; Deisz, Dose & Lux, 1984): a rapid increase in membrane conductance cannot be achieved at a concentration of 100 μM or lower. This difference between the effects of high and low GABA concentrations is consistent with the assumption that the inward current is a manifestation of a Na^+-dependent uptake system which limits the access of exogenous GABA to the postsynaptic receptors (cf. Deisz *et al.* 1984). The difference in the concentration and time dependence of the uptake and conductance mechanisms also explains the biphasic voltage and current responses induced by GABA in the present preparation (see Discussion).

Reversal potential of the putative GABA uptake current

The reversal potential of the presumptive GABA uptake current was examined using brief pulses of 100 μM-GABA in the presence of 200 μM-PTX. The results of experiments of this kind are depicted in Fig. 8, which shows that the reversal potential of this current lies at extremely positive voltages, +30 to +60 mV ($n = 5$). A clear reversal of the PTX-insensitive current could not be achieved in experiments of this kind, but obtaining an estimate of the reversal potential required rather limited extrapolation. When compared in a given neurone, the current induced by 100 μM-GABA in the presence of PTX (i.e. the uptake current) reversed at a voltage level about 100–140 mV more positive than that induced by 100 μM-muscimol under control conditions (i.e. the current mediated by the inhibitory channels; see Fig. 8).

The observations described so far predict that under control conditions (i.e. in the absence of PTX) brief pulses (10 s or less) of a relatively low concentration of GABA (100 μM or less) should preferentially activate the uptake current. This prediction was verified in experiments which showed that 10 s pulses of 100 μM-GABA produced only a small increase in conductance and the reversal potential of the GABA-activated current was rather close to that of the 'pure' uptake current seen in the presence of PTX (Fig. 8). These observations are important since they imply that any estimate of E_{GABA} (when measured in the presence of an operational uptake system) will depend on both the concentration of GABA and on the duration of the GABA pulses used to evoke current responses. In contrast to this, the reversal potential of the current activated by 10 s pulses of 50 μM-muscimol was identical to that observed at 500 μM (four experiments; not shown).

DISCUSSION

An extensive amount of work has shown that an increase in Cl^- conductance plays a dominant role in inhibition mediated by vertebrate $GABA_A$ receptor channels and their counterparts in invertebrate species (Krnjević, 1974; Nistri & Constanti, 1979;

Dingledine *et al.* 1988). A widely accepted assumption is that E_{GABA} is identical to E_{Cl} and, consequently, a depolarizing action of GABA is generally thought to imply active uptake of Cl^- in the target cell. However, there is little direct information on the intracellular Cl^- activity in nerve cells and, therefore, the identity $E_{GABA} = E_{Cl}$ must be viewed as an assumption that calls for experimental testing (see Kaila & Voipio, 1990). It is also noteworthy that in a number of preparations, activation of $GABA_A$-type channels produces biphasic and multiphasic changes in membrane potential (e.g. Barker & Ransom, 1978; Alger & Nicoll, 1979) which are difficult to explain on the basis of a single ionic mechanism.

We have recently shown that in crayfish muscle fibres bathed in a CO_2–HCO_3^--containing solution, bicarbonate is responsible for a large depolarizing component in the GABA-activated total current (Kaila & Voipio, 1987; Kaila *et al.* 1989). Subsequent work on the crayfish stretch receptor neurone showed a similar depolarizing influence of HCO_3^- on the action of GABA. However, a somewhat unexpected finding was that, even in the absence of HCO_3^-, GABA produced a depolarization in most of the preparations examined (Voipio *et al.* 1991; see also Kuffler & Edwards, 1958). Such an effect is not easy to reconcile with the evidence indicating that the SRN is equipped with a K^+–Cl^- co-transport mechanism which maintains an inwardly directed Cl^- electrochemical gradient (Deisz & Lux, 1982; Aickin *et al.* 1982). In the present work, this apparent dicrepancy has been resolved by the demonstration of a sodium-dependent uptake current which contributes to the actions of GABA on membrane potential and current.

Evidence for an electrogenic, Na^+-dependent GABA uptake mechanism in the stretch receptor neurone

That an uptake system limits the access of exogenous GABA to the postsynaptic receptors was postulated in a classical paper on the crayfish SRN by Kuffler & Edwards (1958) who found that the 'inactivation' of bath-applied GABA (observed in the absence of superfusion) could be overcome by stirring the bathing solution. Subsequently, these authors showed that such effects were characteristic of some but not all members of a series of GABA analogues tested (Edwards & Kuffler, 1959). In agreement with these findings, more recent work has shown that manipulations known to inhibit sodium-dependent GABA uptake increase the efficacy of activation of the receptor-gated channels by GABA and those analogues which show the above inactivation or 'fade' phenomenon (Krause *et al.* 1981; Deisz & Dose, 1983; see also Krnjević, 1984). An increase in the efficacy of GABA action brought about by blocking its uptake has also been observed in various vertebrate preparations (Brown & Scholfield, 1984; Rovira *et al.* 1984; Korn & Dingledine, 1986; Akaike *et al.* 1987).

The presence of an uptake system does not only affect the apparent potency of exogenous GABA at the inhibitory channels but it also leads to a concentration-dependent delay in the channel-mediated effects of bath-applied GABA. As shown by Deisz and co-workers (Deisz & Dose, 1983; Deisz *et al.* 1984), with the uptake system in action, the conductance increase induced by GABA at concentrations between 1 and 100 μM develops slowly, reaching its maximum in 20–60 min and an immediate activation of the inhibitory channels is seen at GABA concentrations of 300 μM and higher.

The work cited above does not provide any information concerning the localization of the GABA uptake system. There is evidence that glial cells may play an important role in clearing extracellular GABA at crustacean inhibitory synapses (Orkand & Kravitz, 1971). However, the present results indicate that at least part of the uptake of GABA in the stretch receptor is achieved by a sodium-dependent, electrogenic transport mechanism located in the receptor neurone itself. This conclusion is based on the following observations:

(i) Direct monitoring of GABA-induced net movements of Cl^- showed that the depolarizing action of GABA cannot be due to a current carried by chloride.

(ii) The depolarizing current is inhibited by a sodium-free solution (cf. Kanner & Schuldiner, 1987) and by the GABA uptake blocker NPA (Krogsgaard-Larsen, 1980) but not by the receptor channel blocker PTX (cf. Takeuchi & Takeuchi, 1969; Aickin *et al.* 1981).

(iii) The inward current is activated without delay by low concentrations of GABA (10^{-6}–10^{-4} M).

Direct evidence for the contribution of a current mediated by the Na^+-dependent, NPA-sensitive GABA uptake system on the electrical actions of GABA has not been obtained before in work on any neurone such as the SRN, which receives a GABAergic inhibitory innervation (Elekes & Florey, 1987). In experiments on cell bodies of molluscan neurones, Kehoe (1975) detected an inward current which was activated by various amino acids, including GABA, and most likely reflected the operation of an uptake mechanism. The neurophysiological relevance of these findings was, however, unclear since the cell bodies studied do not receive any innervation, whether inhibitory or excitatory. Furthermore, the putative uptake current recorded in the above study required rather high substrate concentrations (100 μM and higher) and it was activated not only by GABA but also by other neutral amino acids (such as histidine) as well as by acidic amino acids. As pointed out by Kehoe (1975), these findings should perhaps best be viewed as an electrical manifestation of (one or more) less specific amino acid uptake system(s), of the kind present also in a large variety of non-excitable cells (e.g. Schultz & Curran, 1970; Bergman, Zaafrani & Bergman, 1989). In this context, it is of interest to note that preliminary experiments on the SRN have shown that both histidine and lysine fail to activate a detectable uptake current (K. Kaila, B. Rydqvist, M. Pasternack & J. Voipio, unpublished observations). This indicates that the inward current attributable to the GABA-specific uptake is not paralleled by a current mediated by other uptake mechanisms, a conclusion which is in agreement with the complete block achieved by nipecotic acid.

Role of the Na^+-dependent uptake in the bi- and multiphasic effects of GABA on membrane potential

In a number of preparations examined in the present study, the temporal separation of the effects of GABA on the uptake mechanism and on the inhibitory channels was evidently clear enough to result in rather complex response patterns.

The high apparent affinity of the Na^+-dependent uptake mechanism readily explains the biphasic nature of the voltage and current responses frequently observed upon application of 500 μM-GABA under control conditions (e.g. Figs 1 and 6*A*). The initial depolarization (and, in voltage clamp experiments, the initial inward

current component) is evidently due to the immediate activation of the uptake mechanism by GABA. As the concentration of GABA at the postsynaptic membrane keeps rising due to constant superfusion, channel activation takes place as reflected by the transient hyperpolarizing shift (and by the outward current component) which is observed after some delay.

The transient nature of the delayed outward component of the GABA-activated current and of the corresponding negative shift in membrane potential most likely reflects a rapid redistribution of Cl^- ions mediated by the inhibitory channels, as evidenced by the prompt changes in a^i_{Cl} following application of 500 μM-GABA (Figs 2 and 3; see also Adams, Constanti & Banks, 1981). It is noteworthy that, being absent under the present experimental conditions, receptor desensitization (Krnjević, 1981) cannot play any role in the multiphasic responses evoked by GABA.

In some preparations, a depolarizing voltage shift was evident shortly upon the washoff of GABA. Such a depolarizing 'off' response is expected to take place as soon as the GABA concentration in the vicinity of the inhibitory receptors has declined to a level lower than that required for channel activation, but which still is sufficient to activate the uptake. Interestingly, a mechanism of this kind may explain the marked NPA-sensitive rebound of excitability that follows the end of GABA application in the cell body layer of area CA1 in rat hippocampal slices (Rovira *et al.* 1984).

Relationships among E_{GABA}, E_m and E_{Cl}

As pointed out above, prompt activation of the inhibitory anion channels in the SRN requires a relatively high concentration of GABA. Accordingly, we used a near-saturating concentration (500 μM) of GABA to obtain an estimate of the reversal potential of the GABA-activated current under control conditions. Simultaneously, Cl^--selective microelectrodes were used in order to measure the reversal potential of the net Cl^- flux mediated by the GABA-gated channels which, by definition, must equal E_{Cl} (see Kaila *et al.* 1989).

A comparison of the reversal potentials measured in the above manner showed that $E_{GABA} > E_{Cl}$. This result is in agreement with the observation that the GABA-induced depolarization is opposed by a channel-mediated net influx of Cl^- (Fig. 2), i.e. by an outward (hyperpolarizing) current carried by Cl^-. The finding that E_{GABA} is more positive than E_{Cl} (Fig. 3) is most likely due to the electrogenic uptake mechanism. This conclusion gained support from the observation that the current and voltage responses activated at the level of the resting membrane potential by 500 μM-GABA (but not by muscimol) reversed polarity upon withdrawal of sodium. In addition, the current activated by muscimol reversed at a level significantly more negative than E_{GABA}.

A complication that arises from E_{GABA} being determined by the simultaneous influence of channel activation and of the uptake mechanism is that it leads to a concentration dependence of E_{GABA}. Although we did not examine this point in detail, it was interesting to note the brief pulses of 100 μM-GABA (which produced hardly any channel activation) yielded a reversal potential which was almost as positive as that of the pharmacologically isolated uptake current, i.e. the reversal level seen in the presence of PTX (Fig. 8).

A further observation of interest was that E_{Cl} was consistently more negative than

the resting membrane potential. This is in agreement with the operation of a chloride-extruding mechanism (probably a K^+–Cl^- co-transporter) in the SRN (Deisz & Lux, 1982; Aickin *et al.* 1982). In this context, it is also worth noting that the voltage dependence of the resting steady-state level of a_{Cl}^i (Fig. 3*Aa*) may be largely due to a spontaneous quantal release of GABA (Iwasaki & Florey, 1969; Finger & Stettmeier, 1981).

Under experimental conditions similar to the present ones (in the absence of bicarbonate), E_{GABA} in the crayfish opener muscle fibres is very close (within 1–2 mV) to E_{Cl} and GABA does not produce bi- or multiphasic voltage or current responses (Kaila *et al.* 1989). This indicates that, if present in the opener muscle fibres, the GABA uptake current is too small to have a significant influence of E_{GABA}.

In conclusion, the present work shows that an electrogenic uptake mechanism has a profound, direct influence on the current and voltage responses evoked by exogenous GABA in the crayfish stretch receptor neurone. An obvious question that has to be tackled in future work is whether the uptake current contributes to the postsynaptic actions of neuronally released GABA.

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